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Stability and reconstitution of pyruvate oxidase from *Lactobacillus plantarum*: Dissection of the stabilizing effects of coenzyme binding and subunit interaction*

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Abstract

Pyruvate oxidase from *Lactobacillus plantarum* is a homotetrameric flavoprotein with strong binding sites for FAD, TPP, and a divalent cation. Treatment with acid ammonium sulfate in the presence of 1.5 M KBr leads to the release of the cofactors, yielding the stable apoenzyme. In the present study, the effects of FAD, TPP, and Mn^{2+} on the structural properties of the apoenzyme and the reconstitution of the active holoenzyme from its constituents have been investigated.

As shown by circular dichroism and fluorescence emission, as well as by Nile red binding, the secondary and tertiary structures of the apoenzyme and the holoenzyme do not exhibit marked differences. The quaternary structure is stabilized significantly in the presence of the cofactors. Size-exclusion high-performance liquid chromatography and analytical ultracentrifugation demonstrate that the holoenzyme retains its tetrameric state down to 20 μg/mL, whereas the apoenzyme shows stepwise tetramer-dimer-monomer dissociation, with the monomer as the major component, at a protein concentration of <20 μg/mL.

In the presence of divalent cations, the coenzymes FAD and TPP bind to the apoenzyme, forming the inactive binary FAD or TPP complexes. Both FAD and TPP affect the quaternary structure by shifting the equilibrium of association toward the dimer or tetramer. High FAD concentrations exert significant stabilization against urea and heat denaturation, whereas excess TPP has no effect.

Reconstitution of the holoenzyme from its components yields full reactivation. The kinetic analysis reveals a compulsory sequential mechanism of cofactor binding and quaternary structure formation, with TPP binding as the first step. The binary TPP complex (in the presence of 1 mM Mn^{2+}/TPP) is characterized by a dimer-tetramer equilibrium transition with an association constant of $K_a = 2 \times 10^7\ M^{-1}$. The apoenzyme TPP complex dimer associates with the tetrameric holoenzyme in the presence of 10 μM FAD. This association step obeys second-order kinetics with an association rate constant $k = 7.4 \times 10^3\ M^{-1}\ s^{-1}$ at 20 °C. FAD binding to the tetrameric binary TPP complex is too fast to be resolved by manual mixing.

Keywords: coenzyme binding; pyruvate oxidase; quaternary structure; reconstitution; stability

Pyruvate oxidase from *Lactobacillus plantarum* catalyzes the oxidative decarboxylation of pyruvate with simultaneous phosphorylation to give acetyl phosphate, carbon dioxide, and hydrogen peroxide:

$$\text{pyruvate} + P_i + H_2O + O_2 \rightarrow \text{acetylphosphate} + CO_2 + H_2O_2$$

* This paper is dedicated to Professor Robert L. Baldwin on the occasion of his 65th birthday.

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Abbreviations: A, N, and U, acid-denatured, native, and unfolded states, respectively; CDTA, trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; FAD, flavin adenine dinucleotide; TPP, thiamine pyrophosphate; SE-HPLC, size-exclusion high-performance liquid chromatography.

The enzyme was first isolated and characterized by Sedewitz et al. (1984). It is a 265-kDa flavoprotein consisting of four identical subunits (603 amino acids) with tightly bound cofactors FAD, TPP, and a divalent metal ion. At present no crystal structure for the enzyme is available, and a detailed binding mechanism for the cofactors is unknown. Sequence comparison with homologous FAD and TPP binding proteins led to the conclusion that the binding sites for FAD and TPP reside in the C-terminal half of pyruvate oxidase (Schumacher et al., 1991). In contrast to analogous enzymes from *Escherichia coli* (Recny & Hager, 1982) and *Pediococcus* sp. (Götz & Sedewitz, 1991), pyruvate oxidase from *L. plantarum* has been claimed to be TPP independent (Sedewitz et al., 1984): although TPP is known to be essential for the catalytic mechanism, addition of external TPP to the assay mixture has no significant effect on the enzymatic activity of pyruvate oxidase from *L. plantarum*. The enzyme exhibits only limited stability at temperatures beyond 32 °C and pH values outside its pH optimum at pH 5.8–6 (Schumacher et al., 1991). At acid pH, the enzyme loses its cofactors in a reversible fashion; after acid ammonium sulfate precipitation about 20% reactivation could be achieved by Sedewitz et al. (1984).

In the present study, a method for the preparation of apoenzyme was designed that allows the quantitative reconstitution of the active tetrameric holoenzyme. This is a necessary prerequisite for studying in detail the effects of the cofactors FAD, TPP, and divalent cations on the mechanism of reconstitution and on the stability of the enzyme. The main objectives of this study were to determine (1) the spectral properties of holo- and apo-pyruvate oxidase, (2) the state of association of both forms of the enzyme, (3) the effects of bound FAD or TPP on the monomer–dimer–tetramer association equilibrium, (4) the sequence of cofactor binding to the apoenzyme, and (5) the influence of the bound cofactors on the stability of pyruvate oxidase. As a result of detailed reconstitution experiments, we propose a mechanism for the regeneration of native tetrameric holo-pyruvate oxidase from the inactive monomeric apoenzyme. Comparing the results with those obtained for the enzyme from *E. coli* allows an explanation of the observed TPP independence of the *Lactobacillus* enzyme.

In an accompanying paper we describe pyruvate oxidase mutants that are stabilized by single or multiple point mutations (Risse et al., 1992). For the analysis of these proteins, one has to keep in mind that stabilizing point mutations may be effective at the level of the tertiary or the quaternary structure, or by modulating cofactor binding. Furthermore, enhanced stability may result from a combination of all three effects. In this context it is of fundamental importance to examine the nature of the interactions involved in coenzyme binding and the possible synergistic effects in proceeding from the apo- to the holoenzyme.

Results and discussion

Pyruvate oxidase apoenzyme can be completely reconstituted to the native holoenzyme

One of the characteristic features of flavoproteins is that their holoenzymes show considerable spectral differences compared to increments of their apoenzymes plus the free cofactors (Massey & Curtl, 1966). Because a high yield of reconstitution is a prerequisite to investigating the impact of cofactor binding on the structure and stability of pyruvate oxidase, the preparation procedure for the pyruvate oxidase apoenzyme was optimized to allow complete reconstitution of the holoenzyme. Modifying previously reported methods (Strittmatter, 1961; Sedewitz et al., 1984), full recovery of the native holoenzyme could be accomplished through readdition of the cofactors (cf. Materials and methods). Apoenzyme stored at −20 °C showed long-term stability, without significant loss of material due to aggregation.

Addition of FAD or TPP to pyruvate oxidase apoenzyme yields inactive binary complexes

The UV absorption spectra of apo-pyruvate oxidase and the contributions of coenzyme binding to the absorption of the native holoenzyme are illustrated in Figure 1A. The spectra were obtained after equilibrium dialysis of apoenzyme against saturating concentrations of the cofactors. As taken from the positive increments at 280 nm, TPP and FAD contribute to the aromatic absorption in an additive fashion. This suggests that, in the presence of manganese ions, both coenzymes are able to bind to the apoenzyme, forming binary TPP or FAD complexes. The apoenzyme and both binary complexes are enzymatically inactive. Binding of FAD can be determined independently by measuring the absorption in the wavelength range of the coenzyme between 320 and 540 nm. As shown in Figure 1B, the FAD absorption changes significantly upon binding to the enzyme, giving rise to isosbestic points at 335, 376, and 440 nm, with a maximum absorption difference at 406 nm. The spectra of both the binary FAD complex and of the holoenzyme are essentially identical.

In fluorescence emission, FAD binding results in a quench of both the protein and the coenzyme fluorescence (Fig. 1C,D) and, in addition, in dramatic changes in the near-UV CD (Fig. 1G). TPP does not show comparable spectral effects (data not shown).

Pyruvate oxidase holo- and apoenzymes have closely similar secondary and tertiary structures

The backbone structure of apo-pyruvate oxidase is not affected significantly upon coenzyme binding. The far-UV CDs of the apo- and holoenzymes are identical within the

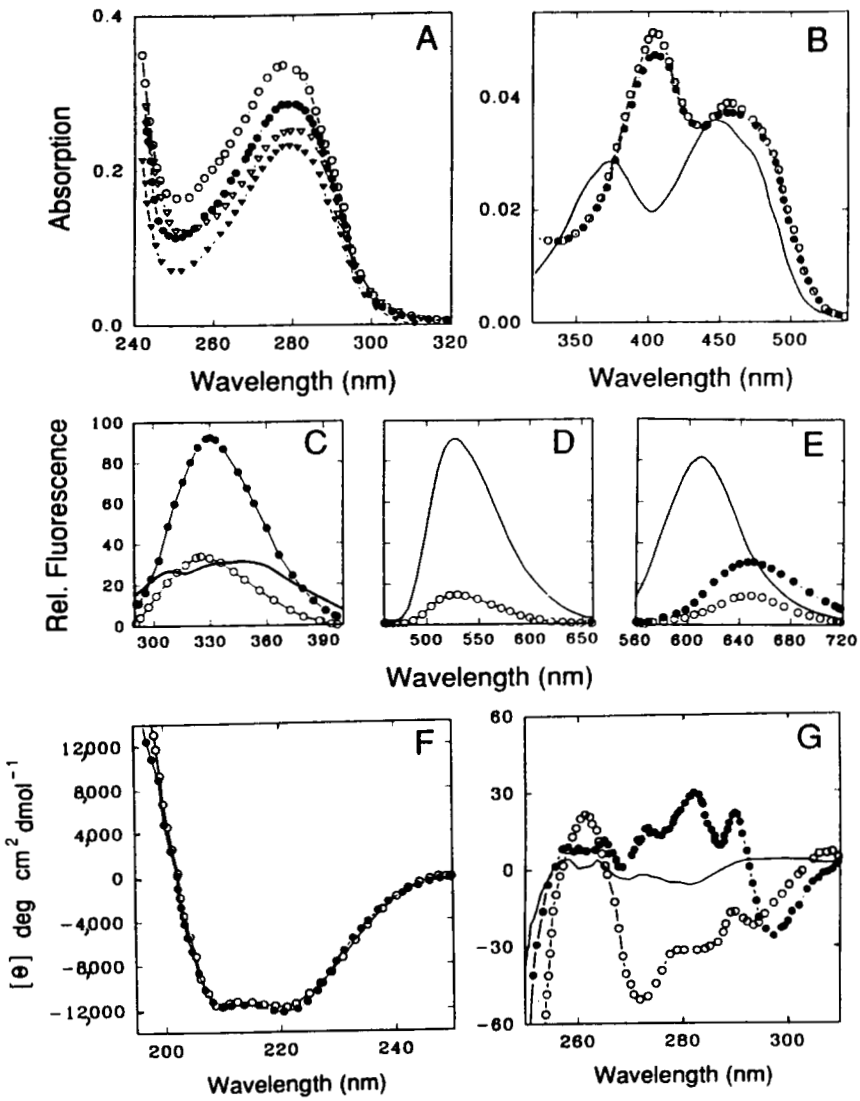


Fig. 1. Spectral characterization of pyruvate oxidase from *Lactobacillus plantarum*. **A:** UV absorption of pyruvate oxidase holoenzyme (○), apoenzyme equilibrated by dialysis (24 h) with 100 μM FAD and 1 mM Mn^{2+} (●), or 1 mM Mn^{2+}/TPP (▽), and apoenzyme alone (▼). Enzyme concentration 2 mg/mL. Spectra were recorded in 0.1-cm cells in 0.2 M potassium phosphate buffer, pH 6.0, plus 20% (v/v) glycerol at 20 °C. **B:** Absorption in the visible range: free FAD (solid line), binary FAD complex (●), and holoenzyme (○). Conditions as in A. **C:** Intrinsic protein fluorescence (λ_{ex} = 280 nm) of apoenzyme (●), holoenzyme (○), and the denatured protein (8 M urea, solid line). Enzyme concentration 0.02 mg/mL. **D:** FAD fluorescence (λ_{ex} = 450 nm): 10 μM free FAD in solution (solid line) and bound to pyruvate oxidase (○). **E:** Nile red fluorescence (λ_{ex} = 550 nm) of apoenzyme (●) at pH 6.0, holoenzyme (○) at pH 6.0, and holoenzyme at pH 3.0 (solid line). **F:** Far-UV CD of apoenzyme (●) and holoenzyme (○). Enzyme concentration 0.2 mg/mL; 0.1-cm pathlength. **G:** Near-UV CD of apoenzyme (●), holoenzyme (○), and the denatured protein (8 M urea, solid line). Enzyme concentration 1.5 mg/mL; 1-cm pathlength. In C–G, 0.2 M potassium phosphate buffer, pH 6.0, 20% (v/v) glycerol plus 1 mM Mn^{2+} at 20 °C was used; in the case of the holoenzyme, 50 μM TPP was added.

limits of error (Fig. 1F). In this context, it is important to note that the spectral properties of the apoenzyme are distinct from those of the denatured enzyme, as is clearly indicated by both intrinsic protein fluorescence and near-UV CD (Fig. 1C,G). Obviously, the release of cofactors does not exert significant effects on the gross structure of the protein. This conclusion is corroborated by dye-binding experiments using Nile red to quantify the accessible hydrophobic surface of the enzyme (Sackett & Wolff, 1987). Binding of Nile red to hydrophobic surfaces of proteins in the denatured and molten globule state causes an increase in fluorescence intensity of the dye, as well as a blue shift of the emission maximum. As illustrated in Figure 1E, release of cofactors from holo-pyruvate oxidase does not cause a drastic enhancement of Nile red fluorescence emission, and the maximum of fluorescence emission (λ_{max}) remains unaltered. The observed marginal increase in fluorescence may be attributed either to dye binding to cofactor binding sites, or to alterations in the quaternary structure. Gross structural changes, as induced upon acid denaturation, are accompanied by significant changes in Nile red fluorescence. In this case, a blue shift of λ_{max} and a drastic increase in fluorescence intensity at 620 nm are observed (Fig. 1E).

The release of coenzymes destabilizes the native quaternary structure

The calculated subunit molecular weight of pyruvate oxidase, based on the amino acid sequence, is 66,177. Determinations of the molecular mass of the holoenzyme by SE-HPLC and analytical ultracentrifugation yield 265 kDa, fully consistent with the tetrameric quaternary structure of the enzyme (Table 1). Correcting for solution viscosity and temperature, the sedimentation constant for the holoenzyme is found to be $s_{20,w}$ = 11.2 ± 0.2S. No concentration-dependent dissociation of the tetramer can

be detected over the whole concentration range covered by sedimentation velocity, sedimentation equilibrium, and SE-HPLC experiments (0.06–1.5 mg/mL) (Table 1).

As shown in Figure 2, the apoenzyme differs from the holoenzyme regarding its state of association. Sedimentation velocity experiments show a biphasic dependence of the sedimentation coefficient on protein concentration. At ≈1 mg/mL, a 6S intermediate is observed that corresponds to the dimer; at higher protein concentrations the state of association approaches the tetramer. The limiting value of the sedimentation coefficient at minimum apoenzyme concentration (4.5S) suggests the monomer to be a compact entity, as compared to the fully denatured enzyme (e.g., in 8 M urea), which is characterized by a sedimentation coefficient of 2.5S (Table 1).

Sedimentation analysis of binary TPP and FAD complex (after 24 h incubation) revealed sedimentation coefficients close to the one observed for the tetramer (Table 1). At low protein concentration (0.06 mg/mL), SE-HPLC analysis of binary TPP complex suggests a dimer–tetramer equilibrium, whereas the binary FAD complex, even at low protein concentration, eluted predominantly as tetramer (Table 1). Divalent cations alone do not affect the association equilibrium (data not shown).

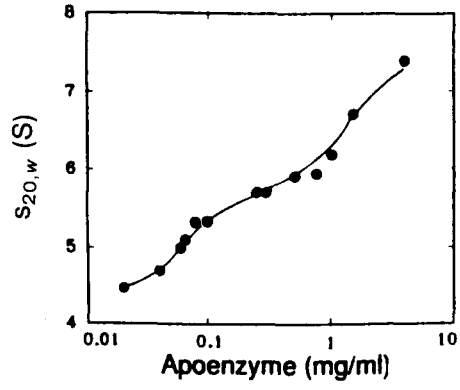


Fig. 2. State of association of pyruvate oxidase apoenzyme. Sedimentation coefficient (in Svedberg units) of apoenzyme, corrected for water viscosity and 20 °C, at varying protein concentration in 0.1 M phosphate buffer, pH 6.0, plus 20% (v/v) glycerol.

FAD and TPP bind synergistically

TPP and divalent cations strengthen FAD binding, at the same time shifting the association equilibrium toward the native (tetrameric) quaternary structure. At high protein concentration (1 μM) the binary TPP complex is predominantly tetrameric (cf. Fig. 5). Assuming four identical and independent binding sites, the stoichiometry of FAD binding to the binary TPP complex is found to be 1 FAD per subunit, with a binding constant $K_a \geq 10^8\ M^{-1}$ (Fig. 3A). In the absence of TPP, dimers and monomers prevail, with monomers as the predominant species at low protein concentrations (cf. Table 1). The dimer and monomer exhibit strongly decreased affinity for the coenzymes, leading to a decrease in the apparent binding constant for FAD (Fig. 3A). Obviously, FAD binding is linked to subunit association.

The influence of Mn^{2+} on FAD binding and subsequent shifts in the equilibrium of subunit association is illustrated in Figure 3B. The protein concentration was varied at constant FAD (10 μM), in the presence of 1 mM Mn^{2+} and in the absence of Mn^{2+}, i.e., at 10 mM CDTA. As becomes clear from the different saturation profiles, the apoenzyme in the presence of Mn^{2+} or Mn^{2+}/TPP binds FAD with high affinity. Removal of Mn^{2+} leads to reduced FAD binding. As mentioned earlier (cf. Fig. 2), increasing concentration of the apoenzyme shifts the association equilibrium from the monomer to the dimer and tetramer, thus favoring FAD binding even in the absence of Mn^{2+}. Consistent with previous findings for the enzyme from *E. coli* (Blake et al., 1982), TPP binding to apo-pyruvate oxidase from *L. plantarum* also depends on the presence of divalent ions (data not shown).

Attempts to determine the role of Mn^{2+} are complicated by the fact that phosphate not only serves as a substrate

Table 1. *State of association of pyruvate oxidase as taken from sedimentation analysis and SE-HPLC*[a]

State of the enzyme	Sedimentation analysis[b]		SE-HPLC[c]
	$s_{20,w}$ (S)	M (kDa)	M (kDa)
Apoenzyme	6.77 ± 0.15	66–132	66
+ 1 mM $MnSO_4$/TPP	11.2 ± 0.3	–	265 ± 132
+ 1 mM $MnSO_4$/100 μM FAD	11.0 ± 0.3	–	265
Denatured (8 M urea)	2.5 ± 0.2	70 ± 5	–
Holoenzyme	11.2 ± 0.3	270 ± 12	265

[a] In 0.2 M potassium phosphate buffer, pH 6.0, 20% (v/v) glycerol, 18–20 °C.
[b] Ultracentrifugal analysis at 1.5 mg/mL initial concentration for the native enzyme and 0.3 mg/mL for the enzyme in 8 M urea.
[c] SE-HPLC analysis at 0.06 mg/mL enzyme concentration.

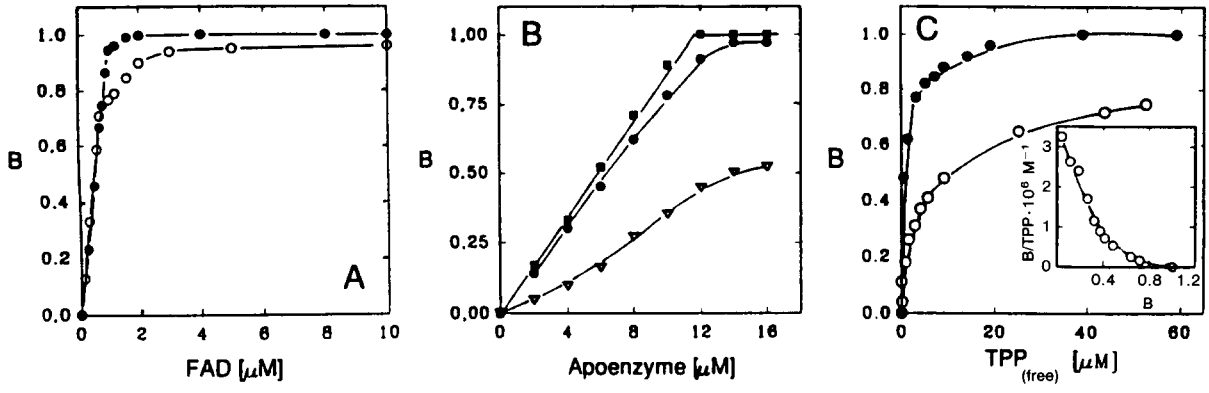


Fig. 3. Binding of Mn^{2+}, TPP, and FAD to the apoenzyme in 0.2 M potassium phosphate, pH 6, containing 20% (v/v) glycerol (20 °C). **A:** FAD binding to 1 μM (subunits) apoenzyme in the presence of 1 mM Mn^{2+}/TPP (●) or of 1 mM Mn^{2+} (○). Separate samples were prepared for each concentration. After 24 h incubation, FAD binding was determined by both fluorescence and regain of activity in the case of holoenzyme reconstitution. The ordinate (B) stands for the ratio of bound FAD per total apoenzyme subunit concentration. **B:** Stoichiometry of FAD binding to the apoenzyme in the presence of 10 mM CDTA (▽), 1 mM Mn^{2+} (●), and 1 mM Mn^{2+}/TPP (■), as determined by fluorescence quenching of 10 μM FAD (λ_{ex} = 450 nm, λ_{em} = 527 nm). The ordinate (B) stands for the ratio of bound FAD per total FAD concentration. **C:** TPP binding to apoenzyme (1 μM) in the presence of 1 mM Mn^{2+}/10 μM FAD (●). Apoenzyme (in the presence of 1 mM Mn^{2+}) was incubated at varying TPP concentrations at 20 °C. After 12 h, 10 μM FAD was added, and, after a further 12 h, activity was measured. TPP binding to apoenzyme (23 μM) in the absence of FAD (○) was derived from TPP equilibrium dialysis experiments at 20 °C (cf. Materials and methods). *Inset:* Scatchard plot of TPP binding data in the absence of FAD. The ordinate (B) stands for the ratio of bound TPP per total apoenzyme subunit concentration.

but also interacts with Mn^{2+} and stabilizes the enzyme. Attempts to replace phosphate by MES or imidazole decreased TPP binding to the enzyme, even at saturating Mn^{2+} concentrations (data not shown). Therefore, TPP binding was measured in phosphate buffer in the presence of 1 mM Mn^{2+}. The apparent binding constant of TPP depends on the presence of FAD (Fig. 3C), complementary to the increased FAD binding in the presence of TPP. As taken from the Scatchard plot given in the inset of Figure 3C, TPP binding in the absence of FAD yields a curved profile, which indicates that the affinity of TPP decreases after binding of one TPP per dimer.

Urea denaturation occurs via a structured intermediate

In order to determine the effects of the cofactors on the stability of pyruvate oxidase, urea- and temperature-dependent deactivation and denaturation transitions were investigated. As has been mentioned, pyruvate oxidase from *L. plantarum* shows long-term stability in 0.2 M potassium phosphate buffer, pH 6, in the presence of 20% (v/v) glycerol plus 0.5–1.0 mM Mn^{2+}/TPP. These conditions were chosen to determine how deactivation, denaturation, and dissociation are correlated in the N → U transition of the enzyme.

As becomes clear from Figure 4A, deactivation and dissociation of the enzyme into subunits and free cofactors precede denaturation. Reversible deactivation of the enzyme at ≈1.8 M urea induces the formation of the apoenzyme. This structure is indistinguishable from the one described for apo-pyruvate oxidase (see above). The data refer to identical solution conditions such that the slight change in the deactivation profile in comparison with both FAD release and subunit dissociation is significant. The difference can be explained by the recombination of FAD with apo-pyruvate oxidase in going from the native tetrameric holoenzyme to its structured constituents, depleted of their cofactors.

Unfolding is a two-step process with transitions at 2.5 M and ≈5 M urea. As a consequence, a structured intermediate is populated at 3–4 M urea. The first unfolding transition is accompanied by a decrease in fluorescence intensity at 330 nm, with λ_{max} almost unchanged. The second transition leads to a characteristic shift of λ_{max} from 330 to 345 nm, corresponding to the maximally unfolded protein. The intermediate still exhibits about 60% of the native far-UV CD signal and tends to aggregate (data not shown).

Because intermediate urea concentrations led to a complete release of cofactors, identical profiles were observed for apo- and holo-pyruvate oxidase beyond 2 M urea. Slight differences in fluorescence emission between apo- and holoenzyme at <2 M urea are attributable to the decrease in fluorescence quenching upon FAD release. Saturation of the enzyme with FAD has a stabilizing effect. Addition of excess FAD (10 μM) shifts the midpoint of the deactivation transition from 0.8 M to 1.5 M urea, whereas TPP at 0.05–1.0 mM concentration has no effect.

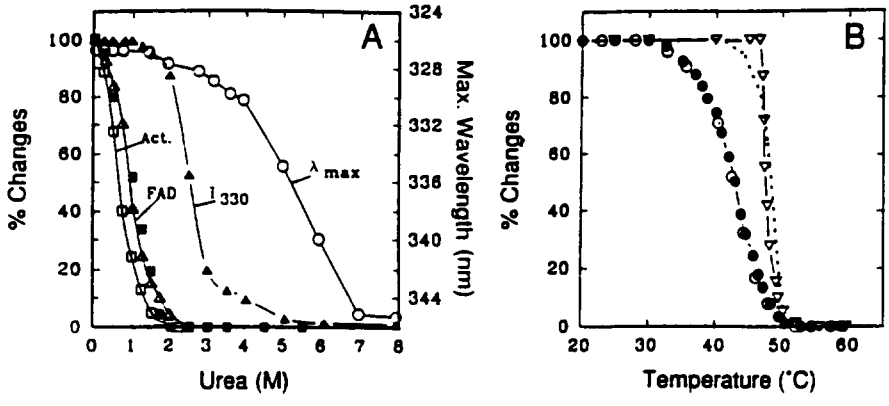


Fig. 4. Urea and thermal denaturation of holo-pyruvate oxidase from *Lactobacillus plantarum*. **A:** Urea denaturation as monitored by activity (□), dissociation (△), FAD release (■), intrinsic protein fluorescence at 330 nm (λ_{ex} = 280 nm) (▲), and fluorescence maximum (λ_{max}) (○). Measurements were performed after 24 h incubation. **B:** Thermal deactivation (○), FAD release (●), and protein aggregation (▽) of holo-pyruvate oxidase. The midpoint of the apoenzyme unfolding transition (dashed line) measured by intrinsic protein fluorescence at 330 nm (λ_{max} = 280 nm) and the aggregation transitions of the apo- and holoenzyme coincide.

Thermal stability of pyruvate oxidase from L. plantarum depends on protein concentration and FAD binding, i.e., on the quaternary structure

Thermal denaturation has often been claimed to cause complete unfolding (Privalov, 1979; Robertson & Baldwin, 1991). In the present case, the fluorescence characteristics at the upper limit of the thermal transition are identical with those of the intermediate observed at 3 M urea. However, in the present case, aggregation may trap structured intermediates, thus preventing complete denaturation. Again deactivation and denaturation do not parallel each other (Fig. 4B). In the presence of 1 mM Mn^{2+}/TPP, release of cofactors and loss of activity precede unfolding and protein aggregation. Apo- and holoenzymes exhibit similar characteristics. Data summarized in Table 2 show that thermal stability of the enzyme depends on the concentrations of both FAD and the protein. Both have been shown to determine the state of association of the enzyme. Conditions that stabilize the enzyme against thermal denaturation correlate with those stabilizing the native quaternary structure. This result clearly suggests that quaternary contacts play a significant role in the stabilization of pyruvate oxidase in its native state.

Pyruvate oxidase apoenzyme regains catalytic activity via consecutive coenzyme binding

In order to confirm the mutual effects of FAD and TPP on the formation of the various ternary complexes of pyruvate oxidase, kinetic reconstitution experiments were performed. Starting from the native apoenzyme, the time course of reactivation was followed, altering the sequence of TPP and FAD addition. As shown in Figure 5, reactivation strongly depends on whether holoenzyme is formed starting from apoenzyme containing either Mn^{2+}/TPP or Mn^{2+}/FAD. Preincubating the apoenzyme with Mn^{2+}/TPP (binary TPP complex) for 24 h, and then starting the reactivation reaction by adding 10 μM FAD, resulted in kinetics with an early burst phase and subsequent slow recovery of full enzymatic activity. Upon simultaneous ad-

Table 2. *Thermal transition temperatures of apo- and holo-pyruvate oxidase*[a]

State of the enzyme	C_{enzyme} (mg/mL)	Transition temperature (°C) determined from: Deactivation	F_{330}[b]	F_{527}[b]	Aggregation
Apoenzyme	0.03	–	48.5	–	48.5[c]
Binary TPP complex (1 mM Mn^{2+}/TPP)	0.03	–	48.5	–	48.5[c]
Binary FAD complex (1 mM Mn^{2+}/0.1 mM FAD)	0.03	–	–	–	50.0[c]
Holoenzyme (1 mM Mn^{2+}/TPP)	0.03	42.0	–	42.0	48.5[c]
Holoenzyme (10 μM FAD)	0.03	53.0	–	53.0	53.0[c]
Holoenzyme (1 mM Mn^{2+}/TPP)	1.50	52.5	–	52.5	52.5[d]

[a] In 0.2 M potassium phosphate buffer, pH 6.0, in the presence of 20% (v/v) glycerol.
[b] F_{330} and F_{527} are transitions measured by intrinsic protein fluorescence at 330 nm (λ_{ex} = 280 nm) and FAD fluorescence at 527 nm, respectively.
[c] Monitored by light scattering at 450 nm.
[d] Monitored by differential scanning calorimetry at a heating rate of 0.5 °C/min (R. Mayer, pers. comm.).

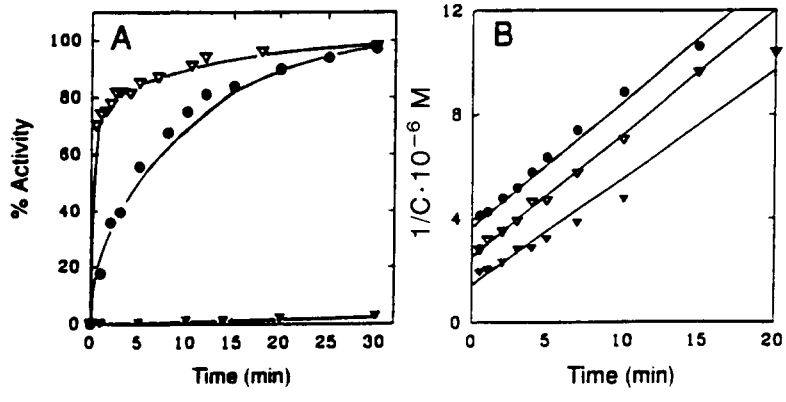


Fig. 5. Kinetics of reconstitution of apo-pyruvate oxidase. **A:** Time course of reactivation of 1 μM apoenzyme in 0.2 M potassium phosphate, pH 6, containing 20% (v/v) glycerol at 20 °C. Addition of 1 mM TPP, after 20 h preincubation of the binary FAD complex (100 μM FAD/1 mM Mn^{2+}) (▼); simultaneous addition of 1 mM Mn^{2+}/TPP plus 10 μM FAD (●); addition of 10 μM FAD after 20 h preincubation of the binary TPP complex (1 mM Mn^{2+}/TPP) (▽). **B:** SE-HPLC analysis of the rate-limiting association reaction starting from the binary TPP complex at varying protein concentrations in 0.1 M potassium phosphate, pH 6, containing 5% (v/v) glycerol plus 1 mM Mn^{2+}/TPP. Peaks were monitored by fluorescence (λ_{ex} = 300 nm, λ_{em} = 330 nm). Cofactor concentrations as in A. The amount of dimer was taken from peak areas; concentrations of the binary TPP complex calculated for the subunits were 0.45 μM (●), 0.9 μM (▽), and 2.7 μM (▼). From the slope in the $1/C_{dimer}$ vs. time plot, a second-order rate constant of $k = 7 \times 10^3$ M^{-1} s^{-1} (20 °C) can be derived; the intercepts with the ordinate ($1/C_0$) allow the initial dimer concentrations to be calculated, yielding an association constant of the tetrameric binary TPP complex of $K_{ass} = 2 \times 10^7$ M^{-1}.

dition of all three cofactors, the initial burst phase is not observed, whereas the kinetics of the rate-determining step are unchanged. In both cases, the half-time of the slow phase of the reaction is ca. 5 min. Starting the reaction from the binary FAD complex (after 24 h preincubation of the apoenzyme with Mn^{2+}/FAD) by adding 1 mM TPP resulted in exceedingly slow kinetics with a reactivation yield of ≈50% after 144 h. Apparently, the TPP binding site is inaccessible in the tetrameric binary FAD complex. Only after FAD dissociation can TPP be incorporated, which slows down the reactivation reaction drastically.

As mentioned, the binary TPP complex exhibits concentration-dependent dissociation. In accordance, reactivation kinetics, starting from the apoenzyme or the binary TPP complex, depend on protein concentration. SE-HPLC analysis of the time course of reassociation revealed that the burst phase during reconstitution of the holoenzyme from the binary TPP complex is due to the preexisting tetramers recombining with FAD very rapidly. This result is confirmed by reactivation experiments starting from the tetrameric binary TPP complex at high protein concentration (1.5 mg/mL). Dilution with buffer containing excess of all three cofactors (1 mM Mn^{2+}/TPP, 0.1 mM FAD), to a final protein concentration close to the one used in Figure 5A, leads to full reactivation within 15 s. Thus, the rate-limiting step in the reactivation of the binary TPP complex at low protein concentration must be the association of inactive dimers to form the native tetramer. As shown in Figure 5B, concentration-dependent kinetics of the dimer-tetramer association of the binary TPP complex, as determined by SE-HPLC, yield a second-order rate constant $k = 7.4 \times 10^3$ M^{-1} s^{-1} at 20 °C. Calculating the initial dimer concentration from the intersections of the straight lines with the ordinate (neglecting the <5% monomeric binary TPP complex), the association constant of the dimer-tetramer transition for the binary TPP complex is found to be $K_a = 2 \times 10^7$ M^{-1}.

Fully denatured apoenzyme is accessible to partial reactivation

Analysis of the recovery of the functional state of oligomeric enzymes after preceding denaturation has been a classical approach for analyzing the mechanism of folding and association (Jaenicke, 1987). In this context, essentially irreversible conditions were found to be well suited for avoiding incorrect aggregation as a competitive side reaction on the folding pathway (Jaenicke & Rudolph, 1986).

In the present case, attempts to regenerate the biological activity of pyruvate oxidase after denaturation in 8 M urea (by rapid dilution to a final urea concentration ≤0.05 M) were unsuccessful. Addition of cofactors to the renaturation buffer did not improve the yield. This holds true also at low protein concentrations, where other proteins usually regain activity. In contrast, renaturation by dialysis in the presence of cofactors may be accomplished with a final recovery of ≈20%, starting from initial urea concentrations between 3 and 8 M. Maximum reactivation, after complete denaturation in 8 M urea, was obtained using a two-step procedure, first diluting the denatured protein in phosphate/glycerol buffer, pH 6, to intermediate urea and protein concentrations of 1–2 M and 40–50 μg/mL, respectively. After 24 h incubation at room temperature, the solution was dialyzed to a final urea concentration of 0.02 M. Under these conditions,

40% reactivation was obtained, independent of the presence or absence of cofactors during renaturation (data not shown).

Conclusions

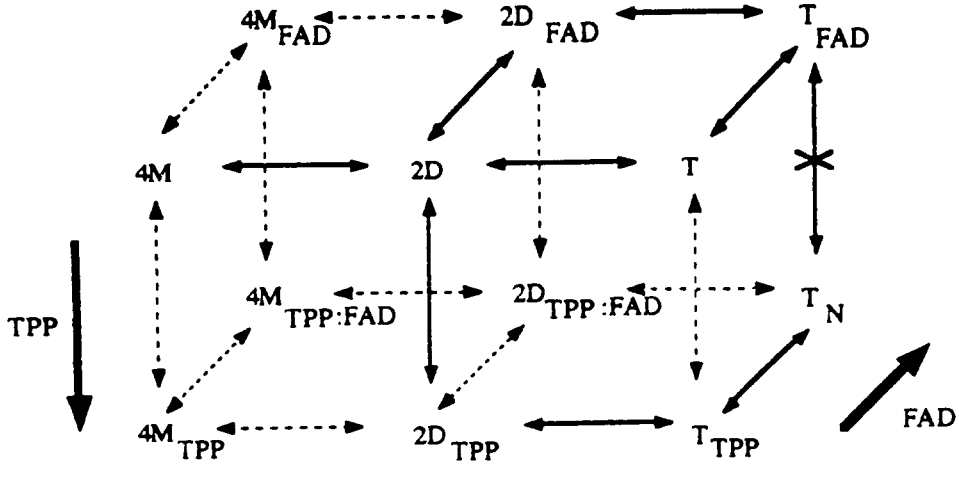
Pyruvate oxidase from *L. plantarum* can be reversibly depleted from its coenzymes, TPP and FAD, by acid ammonium sulfate precipitation. Whereas the secondary and tertiary structures of the apoenzyme are essentially identical to those of the holoenzyme, the state of association differs considerably. In contrast to the holoenzyme, the apo- form of pyruvate oxidase exhibits an association equilibrium M ⇌ D ⇌ T. Binding of FAD or TPP to the apoenzyme shifts the state of association to dimers and tetramers. For the binary FAD complex, tetramers are the predominant species, even at low protein concentration. The respective TPP complex (in the presence of 1 mM Mn^{2+}/TPP) shows a dimer/tetramer equilibrium with an association constant of $K_a = 2 \times 10^7\ M^{-1}$. The binding constants of TPP and FAD depend on the presence of divalent metal ions, with significant mutual effects on their affinity: the apparent binding constants of both coenzymes increase in the presence of the other coenzyme. This observation may be explained by the fact that both coenzymes favor the tetrameric state in an independent way.

In connection with the correlation of coenzyme binding and quaternary structure formation it was of interest to examine how the uptake of TPP and FAD affects the monomer–dimer–tetramer transition. The outcome of the given equilibrium data is a minimum reaction scheme characterized by a consecutive coenzyme binding mechanism with ligand-dependent association/dissociation (Scheme 1). In the first step TPP binds to the monomeric or dimeric apoenzyme. The formation of dimers (D_{TPP}) is a fast reaction, with the first TPP per dimer bound with high affinity. The association to the tetramer represents the rate-limiting step of the reconstitution process. FAD binding, as the final step, influences the dimer–tetramer association equilibrium of the binary TPP complex, leading to the native, fully active enzyme (T_N). In the presence of 1 mM Mn^{2+}/TPP and 10 μM FAD, a rate constant $k = 7.4 \times 10^3\ M^{-1}\ s^{-1}$ was determined for the dimer–tetramer association.

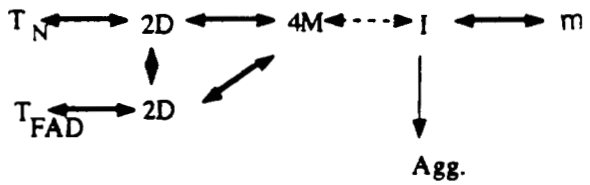
As is clear from the given molecular weight and binding data, full catalytic activity of pyruvate oxidase from *L. plantarum* requires the enzyme to be in its tetrameric state. The thermal deactivation and denaturation transitions show that the transition midpoints depend on the concentrations of both the enzyme and FAD, whereas TPP has no significant influence. The urea-induced deactivation, dissociation, and denaturation pathway is a three-step mechanism (Scheme 2). The deactivation and dissociation into monomers (M) with concomitant release of TPP and FAD make up the first step observed upon urea denaturation. The formation of the binary FAD complex (T_{FAD}) leads to stabilization of the dimeric and tetrameric states. The structured monomers (M) observed at intermediate urea concentrations (1.8 M) are fully reconstitutable to the active enzyme (T_N). Unfolding occurs at higher urea concentrations in a two-step process with an intermediate state (I) at 3–4 M urea. Refolding experiments with fully denatured enzyme (m) yielded, under optimum conditions, 40% reactivation. The low yield of reactivation is caused by the tendency of the intermediate to form aggregates (Agg.).

Previous studies seemed to indicate that pyruvate oxidase from *L. plantarum* is TPP independent (Schumacher et al., 1991). The present data allow this observation to be explained by the fact that bound TPP either interacts directly with FAD or is shielded by FAD due to quaternary constraints.

The compulsory sequential coenzyme binding mechanism is the most important difference in the reactivation mechanism of pyruvate oxidase from the *E. coli* enzyme,



Scheme 1. Minimum reaction scheme for monomer–dimer–tetramer transition upon the uptake of TPP and FAD by the apoenzyme.



Scheme 2. Mechanism of urea-induced deactivation, dissociation, and denaturation pathway of pyruvate oxidase.

where FAD binding and tetramerization *precede* TPP binding (Recny & Hager, 1982). The present results for the *Lactobacillus* enzyme show that FAD and TPP are bound synergistically, which is compatible with either a common binding site or different affinities due to conformational changes accompanying the sequential binding. Proof for either of these explanations has to await the determination of the three-dimensional structure of the enzyme by X-ray analysis, which is currently underway (Y. Muller & G.E. Schulz, pers. comm.).

Materials and methods

Chemicals

Ultrapure urea was purchased from Schwarz/Mann Biotech (Cleveland, Ohio), FAD from Boehringer Mannheim (Penzberg, Germany), and TPP from Sigma (St. Louis, Missouri). The fluorescent dye Nile red was obtained from Eastman-Kodak. All other reagents were analytical grade substances from Boehringer Mannheim or from Merck (Darmstadt, Germany). Quartz-bidistilled water was used throughout.

Pyruvate oxidase

Pyruvate oxidase (5 U/mg protein) from *L. plantarum* (EC 1.2.3.3) was obtained from Boehringer Mannheim. The enzyme was dissolved in 0.2 M potassium phosphate, pH 6, in the presence of 20% (v/v) glycerol, 100 μM FAD, 1 mM TPP, and 1 mM $MnSO_4$ and incubated for 24 h at 4 °C. Then, the holoenzyme was dialyzed in 0.2 M potassium phosphate, pH 6, 20% (v/v) glycerol, containing 0.5 mM Mn^{2+} and TPP to remove excess FAD (2 × 500 mL).

Preparation of apoenzyme

In order to prepare the apoenzyme, the methods of Strittmatter et al. (1961) and Sedewitz et al. (1984) were modified in the following way: 4.3 mg holo-pyruvate oxidase in 600 μL 0.2 M potassium phosphate, pH 6, plus 20% (v/v) glycerol was mixed with an equal volume of 3 M KBr and incubated on ice for 2 min. One milliliter of 50% saturated $(NH_4)_2SO_4$, adjusted to pH 3 with phosphoric acid, was added dropwise under gentle stirring at 0 °C. After further addition of 4 mL of 50% saturated $(NH_4)_2SO_4$, pH 3, the solution was centrifuged for 15 min at 40,000 × *g*. The yellow supernatant, containing free FAD and TPP, was removed, and the pellet was redissolved in 1.2 mL 0.2 M potassium phosphate, pH 6, containing 20% glycerol plus 1.5 M KBr. To accomplish complete removal of the coenzymes, the acid $(NH_4)_2SO_4$ precipitation was repeated a second time. The pellet from the second procedure was redissolved, and the turbid solution was dialyzed against 2 × 500 mL phosphate/glycerol buffer at 4 °C. After spinning down small amounts of aggregated protein (15 min at 10,000 × *g*, 4 °C), the clarified apoenzyme solution was stored at 4 °C or, for long-term storage, at −20 °C.

Determination of protein concentration

The concentration of the pure holoenzyme was determined spectrophotometrically using an extinction coefficient $A_{278nm} = 1.65$ cm²/mg or $A_{406nm} = 0.235$ cm²/mg, estimated from quantitative tryptophan analysis (Pajot, 1976). For the apoenzyme, the extinction coefficient, $A_{278nm} = 1.07$ cm²/mg, was calculated from the amino acid composition (Wetlaufer, 1962).

Assay of enzyme activity

Oxidative pyruvate decarboxylation was determined by oxidative coupling of the reaction product, H_2O_2, with 4-aminoantipyrine in the presence of horseradish peroxidase and sodium 2-hydroxy-3,5-dichlorobenzene-sulfonate. The resulting colored product was measured spectrophotometrically at 546 nm. The extinction coefficient at 546 nm and 25 °C is 16.5 cm²/μmol. The assay mixture contained 0.05 M potassium phosphate, pH 6.5, 10% (v/v) glycerol, 40 mM pyruvate, 2 mM 4-aminoantipyrine, 7 mM 2-hydroxy-3,5-dichlorobenzene-sulfonate, and 0.4 U/mL horseradish peroxidase.

Ultracentrifugation

Sedimentation velocity and sedimentation equilibrium runs were performed in an analytical ultracentrifuge (Beckman Spinco, model E) with a high-sensitivity UV scanning system. Double sector cells (12 mm) with sapphire windows were used at 40,000, 16,000, and 8,000 rpm in an AnG rotor. Meniscus depletion high-speed sedimentation equilibria (Yphantis, 1964) were evaluated using a computer program kindly provided by G. Böhm (University of Regensburg). Data were corrected to water viscosity and 20 °C. In cases where cofactors were present, Philpot-Svensson Schlieren optics and double sector cells (30 mm) were used in an AnE rotor.

Spectroscopy

UV absorption spectra were monitored in a Perkin-Elmer Lambda 5 double-beam spectrophotometer using thermostated quartz cuvettes equipped with a thermistor telethermometer.

Fluorescence emission was measured in a Perkin-Elmer MPF 44A fluorescence spectrophotometer. Excitation wavelengths of 280 or 297 nm for protein fluorescence, and 450 nm for FAD fluorescence were applied at bandwidths of 4 and 10 nm for excitation and emission, respectively. Changes in FAD fluorescence (accompanying the release of FAD from the enzyme) were monitored at 527 nm and corrected for alterations in FAD emission caused by the specific buffers and/or the residual urea concentration. Binding of the fluorescent dye Nile red to hydrophobic surfaces of the protein was recorded using an excitation wavelength of 550 nm. Apo- and holoenzyme (1 μM) were incubated with an equimolar concentration of the dye at room temperature for 15 min in 0.1 M potassium phosphate, pH 6 and pH 3, respectively. Emission spectra in the wavelength range 560–720 nm were corrected for buffer and Nile red.

CD was recorded a room temperature in a JASCO J-500A spectropolarimeter with a 500N data processor at protein concentrations of 0.2 mg/mL in 0.1-cm cells (far UV) or 1.5 mg/mL in 1-cm cells (near UV).

SE-HPLC

SE-HPLC was performed using a Pharmacia Ultrapac TSK 3000 column (7.5 × 300 mm) with a precolumn of 7.5 × 100 mm. All separations were carried out isocratically at 20 °C in 0.1 M potassium phosphate, pH 6, plus 5% (v/v) glycerol at a flow rate of 0.5 mL/min. The column was calibrated with α_2-macroglobulin (M_r = 725,000), β-galactosidase (M_r = 465,000), IgG (M_r = 150,000), Fab (M_r = 50,000), and myoglobin (M_r = 17,800).

Equilibrium dialysis and ligand binding

Ligand-binding experiments were performed by equilibrium dialysis in a rotating Plexiglas block (20 rev/min, 20 °C) containing ten 100-μL dialysis cells. After equilibration (24 h), TPP was determined enzymatically, applying the pyruvate decarboxylase method according to Ullrich (1974).

Urea-induced cofactor release and unfolding

Urea denaturation was measured by fluorescence and CD spectroscopy, as well as by activity measurements and SE-HPLC, at a final protein concentration of 20–30 μg/mL. Deactivation and unfolding in 0.2 M potassium phosphate, pH 6, containing 20% (v/v) glycerol and 0–0.5 mM Mn^{2+}/TPP in the absence and in the presence of 10 μM FAD at 20 °C, were performed by dilution into buffers containing increasing concentrations of urea. To reach equilibrium, final values were measured after 24 h incubation.

Thermal unfolding

Thermal denaturation was performed in 0.2 M potassium phosphate buffer, pH 6, plus 20% (v/v) glycerol in the absence and in the presence of 1 mM $MnSO_4$/TPP and 10 μM FAD. The transitions were monitored by fluorescence emission at 330 nm (λ_{ex} = 280 nm), light scattering at 450 nm, and enzyme activity. FAD release was monitored by fluorescence emission at 525 nm (λ_{ex} = 450 nm). Measurements were performed at 20–70 °C at a heating rate of 0.5 °C/min.

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